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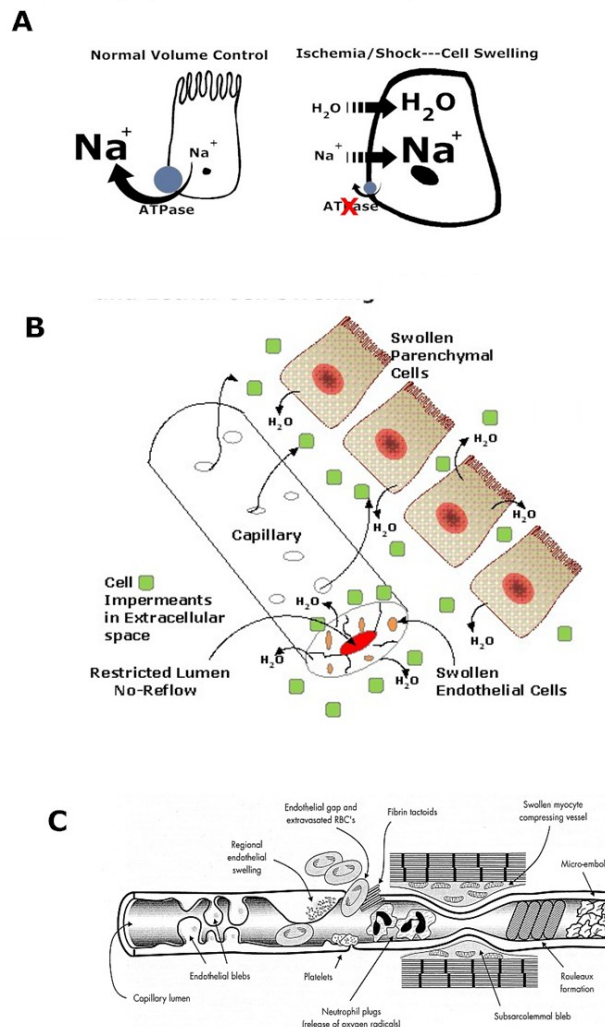
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14. ABSTRACT Severe hemorrhagic shock and trauma on the battlefield have poor outcomes if evacuation and advanced treatment are delayed. The objective of this study was to increase the low volume hypotensive time on the field by using cell impermeants in the low volume resuscitation (LVR) solution. The hypothesis is that local tissue ischemia due to shock causes significant cell swelling in vital tissues and that alleviation of cell swelling with early administration of cell impermeants prevents this, which increases the golden hour on the field and survival after full resuscitation at the forward hospital. To test this hypothesis, first, the impermeant effects of a family of agents were tested in a liver slice ischemia model in-vitro. All impermeants prevented tissue slice cell swelling after 1 hour of hypoxic tissue culture, which was proportional to the concentration and molecular weight of the impermeant. The best timing was to administer impermeants after the start of ischemia and not after reperfusion (resuscitation). Next, anesthetized rats were shocked to a pressure of 30-35 mm Hg until their lactate rose to 10 mM, then they were given a saline low volume resuscitation solution, then fully resuscitated when their lactate rose to 10mM again, and finally recovered for 24 hrs. Impermeants given into the LVR solution significantly increased the LVR time, which is the time between administration of the LVR solution and full resuscitation. This is a marker of tolerance to the low volume period (golden time). Gluconate (10-15%) in the LVR solution produced the best individual protection. Mixtures of trehalose, raffinose, gluconate, and sorbitol at a specific ratio was optimal. This mixture doubled the LVR time and significantly improved cardiovascular, hepatic, renal, and metabolic function during the LVR period and 24 hrs after full resuscitation. Gluconate seems to produce salutary effects beyond what is attributable to its impermeant effects.. In conclusion, severe hemorrhagic shock causes significant cell swelling which contributes significantly to resuscitation injury. Inclusion of cell impermeants in LVR crystalloids significantly increases the golden hour on the field and improves multiple organ function and survival later.					
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INTRODUCTION: The direct and indirect effects of severe and prolonged tissue hypoxia due to hemorrhagic shock are the leading cause of death for battlefield injuries (1,2). Resuscitation in the field is often seriously inadequate even if the patient makes it to the local field hospital for full resuscitation. The delay to evacuation in far forward units could take many hours to days so time becomes critical and maintaining patients in a low volume status for long periods is a real possibility (3). Work done in this proposal will significantly increase this time period. Past resuscitative attempts have focused on maintaining tissue perfusion and oxygen delivery to rebalance the oxygen supply-to-demand ratio by administration of large volumes of crystalloid. However, it is now recognized that controlled resuscitation using lower volumes is effective, more economical, and essential for battlefield operations (4). The objective should be to restore as much oxygen delivery in the lowest volume as possible only to patients that will benefit from the therapy. This approach can be improved by using agents in the low volume resuscitation solution that preserve tissue perfusion. One significant impediment to tissue perfusion during low pressure states is no reflow in the microcirculation caused by cell swelling induced by prolonged cellular ischemia. Endothelial cells in the capillaries swell during ischemia and shut off flow through already narrow capillary corridors. Parenchymal cell swelling further compromises the microcirculation by compressing the capillary from the outside. The significance of this proposal is that it uses proven and tested technology and solutions developed for organ preservation for transplantation that resolve ischemia-induced cell swelling and applies it to shock and resuscitation injury. Specifically, the use of simple cell impermeants in low volume resuscitation solutions will significantly attenuate cell swelling, capillary no reflow, and preserve DVO_2 to critical tissues both before and after full resuscitation. This lessens end organ failure and improves survival since distribution of the limited oxygen availability is enhanced. Additionally, cell swelling per se is lethal to tissues so reversal with impermeants is salutary. Thus, attacking one of the root causes of severe resuscitation injury (cell swelling) using proven methods should have a cascading effect that improves the survival of military personnel from severe hemorrhagic shock and resuscitation injury. These concepts and products will also be useful in civilian treatment of shock. The significance of this approach, beyond the obvious medical benefits to humans, is the simplicity of the concept, its proven track record of use in organ transplantation, and the extreme stability of the active components (cell impermeants). The diagram below summarizes the biological problem and the solution.

Mechanisms of Ischemic Cell Swelling and the Biophysical effects of Cell Impermeants



BODY:

The objectives and specific tasks for the first year were to;

1. Characterize the performance of various potential cell impermeants that may be used in this study in an in-vitro tissue (liver) slice model of ischemia-induced cell swelling
2. Define the attributes of a model impermeant, gluconate, and others in a rodent combat model of severe hemorrhagic shock with low volume resuscitation and survival.

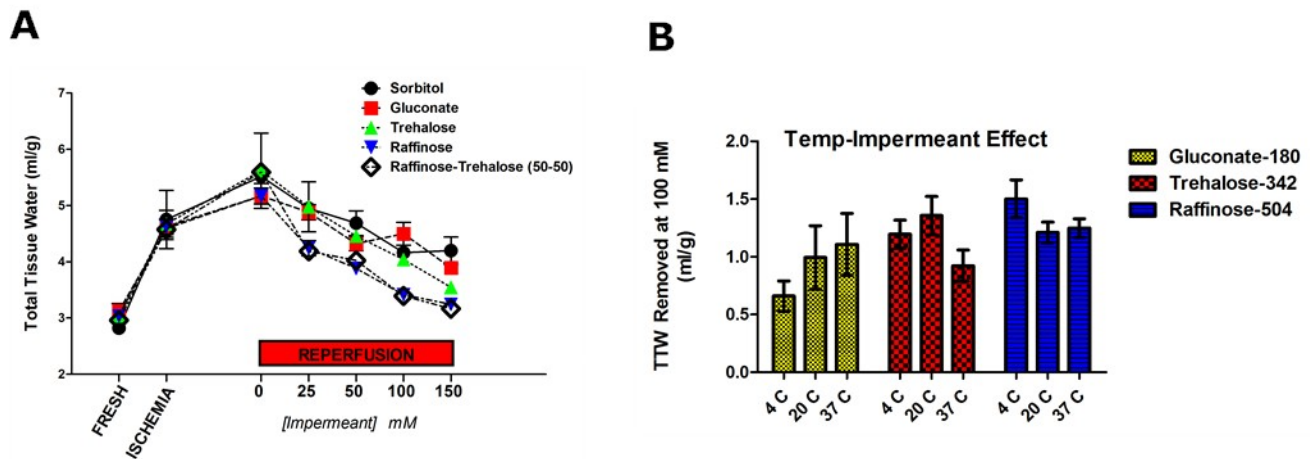
The following experiments and their results were used to address those objectives and tasks.

I. **In-Vitro Cell Impermeant Studies:** Cell impermeants are small molecules that are large enough to freely transit across the capillary wall but are too large and / or too charged to enter the cell. Thus, cell impermeants preferentially load into the extracellular space in equilibrium with the capillary space where they serve to pull water out of the cell. More accurately, they prevent water from moving into the cell secondary to ionic shifts in the cell caused by ischemia-induced loss of ATP dependent volume control mechanisms (Na/K ATPase or sodium pump failure). An important attribute of cell impermeant molecules is that they are relatively non-toxic, which is necessary since large amounts are required for biological activity. A concentration of between 60-100 mM in the extracellular fluid compartment is generally needed to prevent cell swelling secondary to ischemia (5). The molecules tested were:

- Sorbitol
- Gluconate
- Trehalose
- Raffinose

These agents have been extensively used in organ preservation solutions so their safety and efficacy in vital organs is well known and established (5-8). In this experiment, mouse livers were excised from anesthetized mice. Slices of liver were prepared in the cold with a Stadie-Riggs microtome to provide a uniform thickness of less than 0.5 mm. Slices were placed in 25-ml Erlenmeyer flasks filled with 1.5 ml of Krebs buffer and incubated for various times in a Dubanoff metabolic incubator at 37° C. Control slices were incubated under an atmosphere of oxygen while ischemic hypoxia was induced under 95% nitrogen and 5% CO₂. Some slices were subjected to 1 hr of ischemia and one hour of oxygenated reperfusion. At various times, some slices were removed from the incubator bath and the wet:dry weight ratios were determined to calculate total tissue water (TTW) and cell swelling. **Figure 1** shows the results of these studies when various concentrations of cell impermeants were added during the ischemia time only.

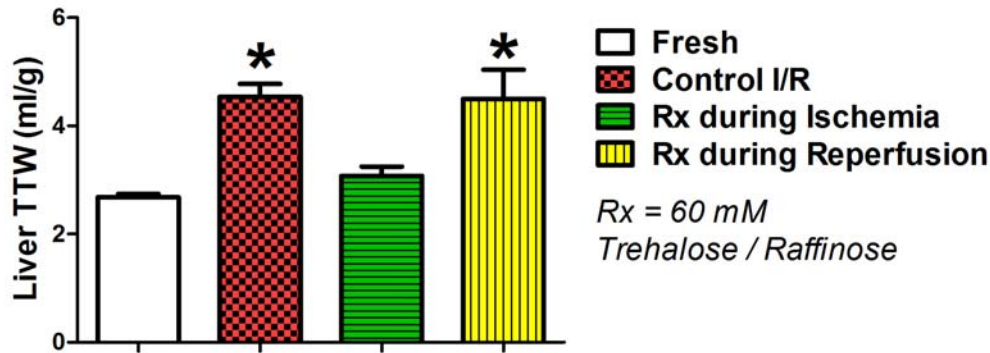
Figure 1. Properties of Cell Impermeants on Water and Volume Control in Ischemia



From these data, we conclude that under in-vitro ischemic hypoxia conditions in liver tissue, ischemia-induced cell swelling is reversed by cell impermeants proportional to their extracellular concentration and their molecular weight. Furthermore, gluconate, a smaller impermeant, shows some temperature dependency while the larger impermeants (trehalose and raffinose), do not.

Next, we explored the timing of cell impermeants during ischemia. Specifically, we explored if cell impermeants are active during the ischemia period or during the reperfusion period. This is vital to know if they are to be transitioned to clinical use in battlefield shock since the timing dictates when they can effectively be used. **Figure 2** shows the timing dependency of cell impermeants in the tissue slice model.

Fig 2. Impermeant Timing



Clearly, the most effective time to deliver cell impermeants to prevent ischemia-induced cell swelling in this model is DURING the ischemia period and not when reperfusion is occurring. Clinically, this means administering cell impermeants during shock and during the low volume state, but not during resuscitation. This fits rather well with the paradigm of battlefield low volume resuscitation where limited amounts of crystalloids are used to help maintain soldiers on the field until evacuation and more definitive resuscitation can occur at a forward field hospital. We see the low volume resuscitation fluid more as a vehicle for drug delivery to shocked soldiers than as volume replacement to increase DVO₂. The low volume resuscitation solution used by combat medics will serve as the perfect vehicle to deliver cell impermeants on the field. This will increase capillary perfusion by reducing ischemia-induced cell swelling. The use of these agents in LVR solutions is attractive because they are generally very stable under harsh austere conditions experienced on the battlefield.

II. **Rodent Shock Studies:** Once the feasibility of these impermeants in preventing cell swelling in ischemic tissues was established in-vitro, we explored the use of these agents in intravenous LVR solutions in combat models of hemorrhagic shock and trauma.

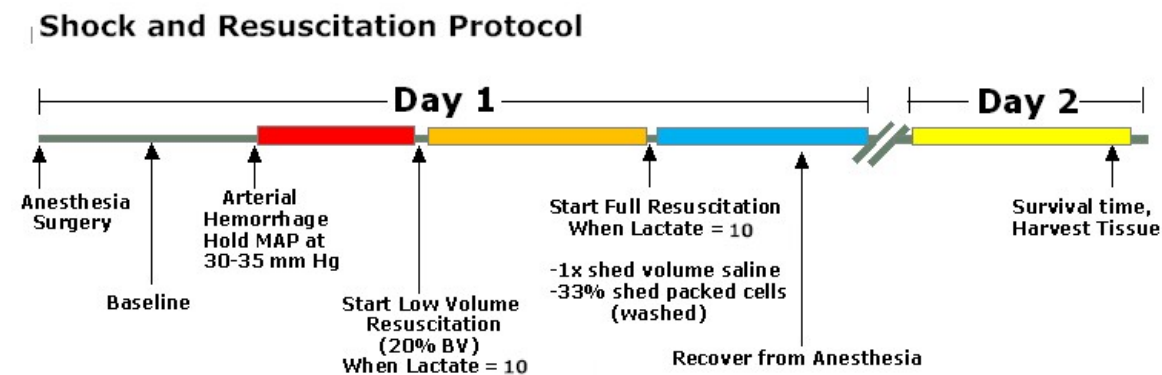
Table 1. Calculated increase in the extracellular osmolarity after administration of LVR solutions containing various concentrations of test cell impermeants

Cell Impermeant	Molarity (mM)				
	25%	20%	15%	10%	5%
Sorbitol	122	98	73	49	24
Na-Gluconate	102	82	61	41	20
Trehalose	65	52	39	26	13
Na-Lactobionate	62	50	37	25	12
Raffinose	44	35	26	18	9
PEG-8K	2.8	2.2	1.7	1.1	0.6

% are the impermeant concentrations used in the LVR solution. Values estimated for a 350g rat with; ECV=49 ml, Blood vol.= 21.8 ml, LVR volume = 4.35 ml. Impermeants in the shaded area are hypothesized to be therapeutic, based strictly on osmotic calculations.

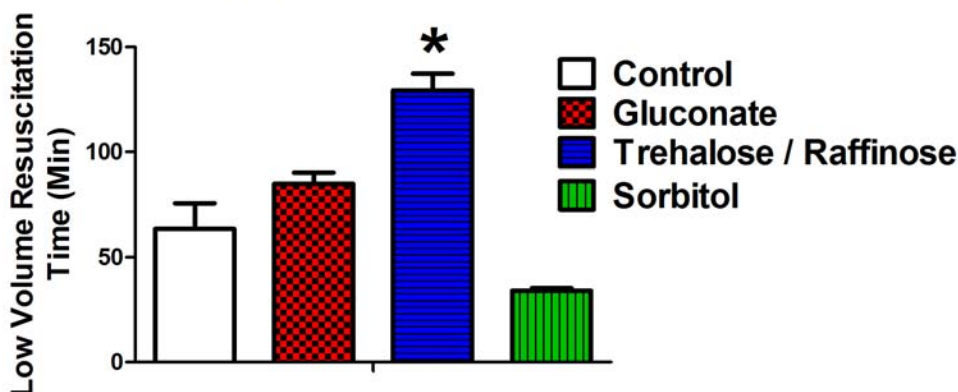
To make this work, large amounts of cell impermeants must be solubilized in small volumes of solution in order to raise the impermeant concentration in the interstitial fluid compartment to about 60 mM. Table 1 shows the predicted impermeant extracellular osmolarity when various molecular species of cell impermeants are added to LVR solutions. Keeping in mind that the target concentration of 60 mM is needed, the LVR solutions become rather concentrated, especially with the high weight agents. These amounts have never been used before for this purpose. So even though we know they work in isolated tissues at these high concentrations, the systemic effects are largely unknown and untested. To answer these questions, a rodent model of hemorrhagic shock was established and tested for use in these studies.

Adult Sprague-Dawley rats were anesthetized with isoflurane and bled to a mean arterial pressure (MAP) of 30-35 mm Hg and held there until the plasma lactate rose to 9-10 mM. At that point, the low volume resuscitation was started by infusing a volume of saline equal to 20% of the animals calculated blood volume over 10 minutes. This drives down the lactate, increases blood pressure, and temporarily restores partial DVO₂. However, as the low volume saline third spaces, the lactate again rises and MAP falls (9). When the lactate again reaches 9-10 mM, full resuscitation is started with a volume of saline equal to the shed blood volume and containing 33% of the shed red blood cells (washed). The time from the start of the LVR until then time of the full resuscitation is termed the LVR time, which represents the tolerance of the subject to the low volume state. In clinical terms, this represents the maximum amount of time a patient can safely remain in the low volume state until they absolutely require full resuscitative care. It is an index of what has been termed the golden hour in shock. In fact, the LVR time in most of our control animals is about an hour. The diagram below shows a schematic of the shock protocol used for these studies.



In the lactate controlled LVR model of hemorrhagic shock, the use of impermeants significantly increases the LVR time as shown in **Figure 3**.

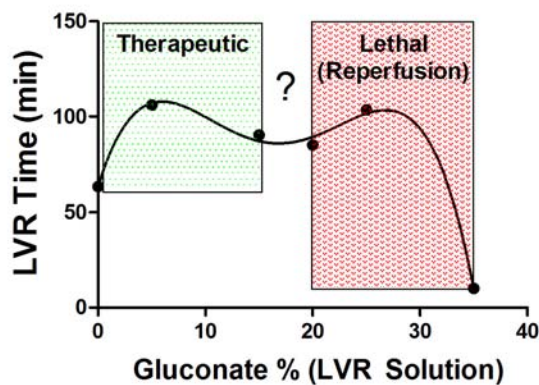
Fig 3. Low Volume Resuscitation Times
(Lactate Controlled)



In these studies, the untreated LVR time was about 60 min. The use of a 50/50 mixture of trehalose and raffinose doubled the LVR time to about 120 min. This indicates that the shocked subjects are able to tolerate the low volume state twice as long when they receive cell impermeants than when they receive saline alone. Their safe time in shock on the field has now effectively doubled by the impermeants, which should mean greater chances of successful evacuation and better performance of the patient when they finally are evacuated and receive full resuscitation. These models are complex, chaotic, and non-linear descriptions of the state of traumatic shock and so are quite variable. Brute force of numbers and repetition is necessary to see changes. The response of sorbitol in this study is an artifact of its unique biochemistry. Specifically, sorbitol, unlike the other impermeants tested, is produced in the mammalian cell as an osmolyte from glucose by aldose reductase, which can be converted back to glucose by sorbitol dehydrogenase (10). Since sorbitol is the smallest of the impermeants, its rate constant for entering the cell is the fastest and some does enter cells, especially more permeable cells like hepatocytes. Once in the cell, it can be converted to glucose and anaerobically fermented under ischemic conditions during shock to lactate. The lactate produced by sorbitol loading during ischemia elevates the plasma lactate faster than normal, which in this study, triggers an artificially short LVR time because of the mechanics of the protocol. Essentially, sorbitol complicates the experimental design if used at high concentrations. Therefore, the short LVR time seen in this study with sorbitol (Fig 3) is not an accurate reflection of its use in shock, but rather a peculiarity of its biochemistry.

The use of impermeants in shock is driven by loading enough osmolytes into the interstitial space to be effective while avoiding toxic effects at the higher concentrations. Since nobody knows what those relationships are, we conducted experiments looking at a dose response effect of a single impermeant (gluconate) using our rodent shock model. Gluconate was chosen because its use in LVR solutions is helpful above 10% and it may be the most likely to have secondary biological effects independent of its

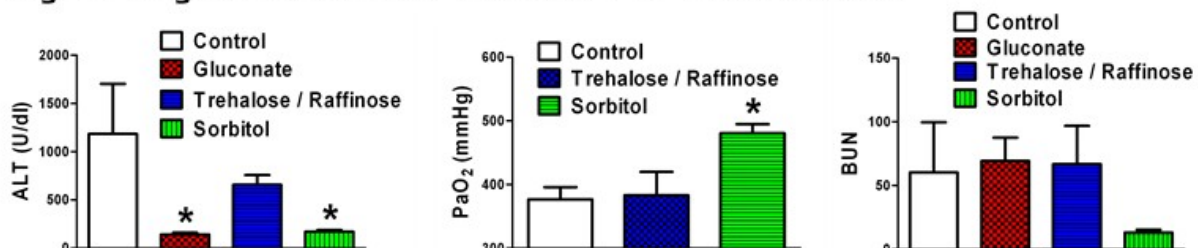
Fig 4. Gluconate Dose-Response



cell impermeant effects, based on our experiences in organ preservation. This relationship is shown in **Figure 4**. The salutary effect of gluconate, as indexed by the increase in the LVR time in the shock model, was most prevalent between 7-15% by weight in the LVR solution. Concentrations above 20% were often counterproductive. Gluconate is a good chelator (11), which may partially explain its toxic effects at high concentrations. On the other hand, chelating divalent cations like calcium with gluconate during ischemia likely provides some added value by protecting the mitochondria.

In the shock model, we typically recover the animals after full volume resuscitation and they are allowed to recover for 24 hours. After 24 hours, the animals are re-anesthetized and multiple organ systems are evaluated for function. These results are seen in **Figure 5** for the different impermeant species.

Fig. 5. Organ Function 24 hrs After Full Resuscitation



Gluconate and sorbitol had significant protective effects on liver cell injury after shock and resuscitation while sorbitol protected the kidneys and lungs too. The histological effects of these agents during shock has not yet been analyzed but the samples have been collected. Trehalose and raffinose, while being better biophysical cell impermeants than gluconate and sorbitol, often show less effectiveness in end organ function on the second day. It seems from these studies that impermeants act differently and each has some unique salutary effects in shock. The concept of developing an optimized formulation that is based on all of the attributes of each molecule tested individually makes sense. This was investigated next.

III. **Optimized LVR Solution:** The previous cell and rodent studies using both systematic and iterative experimental techniques has led to the development of an “optimized” cell impermeant based low volume resuscitation solution. The formulation is based on:

- **Gluconate 10%**
- **Raffinose 5.75%**
- **Trehalose 4.15%**
- **Sorbitol 2.5%**

This solution delivers a total of 1,265 mOsm/l of impermeant pulling power and achieves an impermeant concentration in the interstitial space of over 60 mM, which is our target. Testing this solution has just begun and is a major objective for the next year. Testing was started in a paired model in an attempt to wring out as much biological and chaotic variability as possible. In this design, a control animal receiving saline is done first. The LVR time for that animal is used for the paired companion animal done at the same time, but treated with the impermeant cocktail. This was done because LVR times, biological responses to shock, and overall health of the animals varies considerably but predictably according to the calendar and season. So it was hoped that animals paired-in-time would control for this variability. This seems to work. The preliminary data is shown in **Figure 6** and **Figure 7**.

Figure 6. Optimized Impermeant Effect on Hepatic and Renal outcomes 24 hrs after Resuscitation

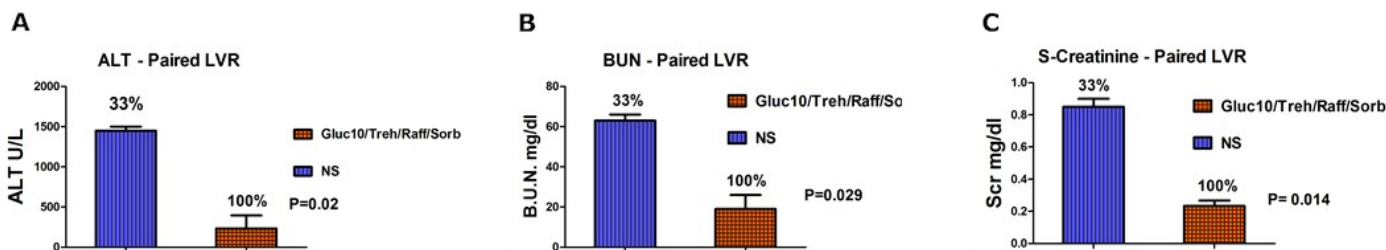
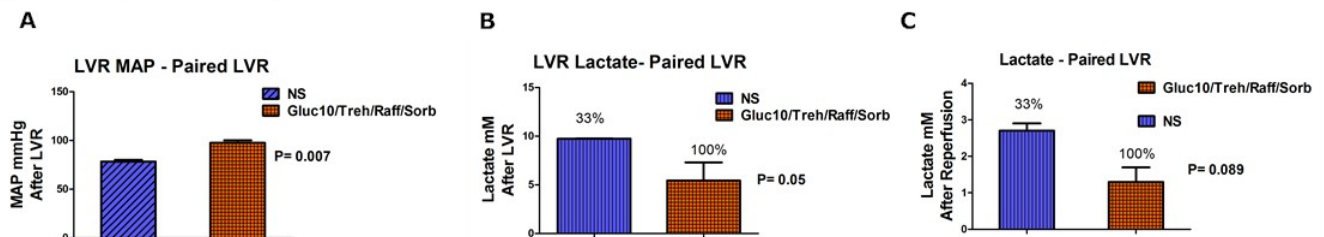


Figure 7. Optimized Impermeant Effect on Arterial Pressure and Metabolic Outcomes 24 hrs after Resuscitation



With only about 3 animals in each group, a significant difference in organ and metabolic function is still evident between the control and the impermeant treated group. In all cases, improvements in organ and metabolic function were seen 24 hours after resuscitation when cell impermeants were used. The goal is to extensively test this formulation in the rodent model this year and add an oncotic agent to the formulation to accelerate water flux from the tissue. Then we will transition the final prototype over to the porcine shock and critical care model for preclinical evaluation in our large animal trauma ICU in the third year.

KEY RESEARCH ACCOMPLISHMENTS:

The following significant research accomplishments include:

1. Characterization of the cell impermeant properties of a family of potentially clinically useful cell impermeants in an in-vitro model. These properties were tested in response to warm ischemia-induced cell swelling
2. Determination of the temperature dependence of cell impermeants in preventing ischemia-induced cell swelling
3. Determination of the optimal timing of administration of cell impermeants to prevent ischemia-induced cell swelling
4. Testing of various molecular species of cell impermeant in a combat model of hemorrhagic shock and trauma utilizing the low volume resuscitation concept
5. Describing the sorbitol effect on lactate production
6. Determining the individual attributes of each cell impermeant.
7. Determine the LVR times and the end organ function 24 hours after resuscitation in the military shock model and the effects of cell impermeants on these values.
8. Perform a dose-response relationship of gluconate with shock outcomes (LVR time)
9. Formulate an optimized cell impermeant cocktail based low volume resuscitation solution
10. Begin testing of the optimized solution in the combat shock model

REPORTABLE OUTCOMES:

1. Presentation of interim results at the DoD Hemorrhage and Resuscitation Research and Development Program, Metabolic and Tissue Stabilization Research In-Progress Review (IPR), 12-13 June 2013
2. Production of a manuscript for later submission for publication (Not appended since it is currently still in draft form)
3. Submission of an Invention Disclosure at VCU (before the project started) and submission of a US patent application (after project started)
4. Meetings set with the FDA on a plan for approval (510K or IND), Washington DC, 2013.

CONCLUSIONS:

1. Cell swelling significantly occurs in liver tissue in response to warm ischemia
2. Cell impermeants prevent ischemia-induced cell swelling proportional to the concentration and molecular mass of the impermeant
3. Cell impermeants are only effective when they are present at the time of ischemia (shock) and not during reperfusion (resuscitation).
4. Multiple species of cell impermeants given into the LVR solution significantly prolongs the LVR time and increase the tolerance to the low volume state. Therefore, cell swelling plays a significant role in tolerance to low flow states
5. Cell impermeants improve organ function 24 hours after resuscitation. Therefore, cell swelling plays a significant role in multiple organ dysfunction following prolonged hemorrhagic shock and resuscitation
6. Cell impermeants, like gluconate, have an optimum performance concentration of about 40-100 mM in the extracellular fluid compartment. Higher concentrations are generally counterproductive.
7. Multiple cell impermeant species can be formulated at specific ratios that produce optimal effects on resuscitation injury when administered in LVR solutions
8. Impermeant based LVR solutions are beneficial in military-style shock scenarios.

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